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13. SUPPLEMENTARY NOTES

14. ABSTRACT. In this project, we postulate that tumor-specific T cells could be used to produce an immunotoxin (IT) targeting tumor cells only when these T cells are specifically activated by the tumor.
We have engineered l entiviral vectors to modify tumor specif ic T cells with our immunotoxin. PEA based immunotoxins affect cell viability by ADP ribozilation of their elongation factor - 2. Indeed, we have also generated a pro ducer cell line resi stant to PEA in ord er to produce high titer of vector encoding the IT. We have first used cell lines to demonstrated that the IT could be produce d, secreted, and th at this secreted IT has a specific lethal activity toward CD22 target cells. We have then protected our T cells against the possibl e effects of endogenous IT and c haracterized several of their functions and phenotypes. Al together, o ur data revealed that IT -producing tumor-specific T cells and IT -producing non specific T cells (OKT3-blast) efficiently produce IT and the IT secreted sp ecifically kills CD22-tumor cells. Our data also showe d that the IT released (by T cells) does not impair T cells original killing potential. Thus by allowing a double killing mechanism , our IT potentiates the eradication of CD22-tumors by T cells.

15. SUBJECT TERMS
Immunotoxin, tumor specific T cell, T cell activation, CD40L, antigen specific, Elongatio n factor

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INTRODUCTION.

Researchers have succeeded to enhance the effector function of monoclonal antibodies by coupling toxic moieties to the targeting portion of the antibody. These "warheads" have included radionucleotides and toxins (immunotoxins: IT). The antibody binds to the cell surface and the toxin is internalized into the cytosol, where it inhibits critical cell functions or damages the cell membrane, leading to cell death. The commonest IT are composed of the variable domain of a monoclonal antibody single (scFv) or double chain (dcFv), conjugated or fused via a linker sequence to a toxin that has been modified to decrease nonspecific binding to non-targeted normal cells. Although this approach has shown promise in animal models, toxicity issues have limited its clinical application. Only a small proportion of the drug reaches target cells after systemic injection, meaning that relatively high doses of IT are required to induce a significant biologic effect. Consequently, the increase in killing of target cells mediated by toxin is partly offset by increased toxicity. Non-specific clearance by liver and kidneys in particular may produce substantial and even fatal damage to these organs at doses optimal for anti-tumor activity. In hematological malignancies, for example, systemic injection of IT may cause vascular leak syndrome, thrombocytopenia, and liver damage. Here, we propose to minimize this toxicity by using tumor-antigen specific T cells to further target delivery of an immunotoxin, the *CD22-Pseudomonas exotoxin A* (CD22-PEA), which has already been used in a clinical setting. The toxin portion contains the translocating and ADP-ribosylating domains of PEA, and the native cell-binding portion is replaced with a CD22 scFv that directs targeting to B lymphocytes. CD22-PEA was tested in a Phase I trial in B-cell malignancies, but tumor responses, particularly in hairy cell leukemia, were offset by an unfavorable toxicity profile. The current project will use the anti-tumor activity of CD22-PEA while minimizing its adverse effects by delivery from T cells. Because the T cells we use are specific for tumoral antigen, they can only be activated when they encounter the tumor. By controlling the IT production with a promoter dependent on T cell activation, and using tumor specific T cells, we can limit the production and delivery of IT to the tumor site. This approach should increase the quantity of IT delivered to the tumor while preventing toxicity to the normal tissue.

BODY.

This is a final progress report for Aim 1: To generate cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigens LMP1 and LMP2, and to engineer these lymphocytes to produce an anti-CD22-toxin following T cell activation (using CD40L promoter).

We first constructed various vectors that produce IT under the CD40L and the CMV promoters. We succeed to protect our produced cell lines and T cells lines form the lethal effect of the IT, succeed to produce IT-viral vectors, and succeed to efficiently transduce T cell lines.
Next, we have generated CD3-activated T cells lines (named OKT3 cells), transduced them with the IT vector, expanded them, and shown that their native phenotype and function were maintained.
Then we have shown that these transduced T cells were not malignant (their proliferation depends on IL-2 and withdrawal of IL-2 induces their cell death). We also showed that their phenotype was unimpaired as the level of important surface molecules (including CD3, CD4,

CD8, chemokine receptors, adhesion molecules, and activation marker (CD25)) were similar in both parental and modified T cells.
We also confirmed that IT-producing T cell functions were preserved. Indeed, the results from proliferation assays and testing of IL-2 and IFN-γ production show equal proliferation and cytokine production for both parental and modified T cells. This corroborate with the analysis of CD25 expression previously observed with T cell activation.
Furthermore, similar migration confirmed the presence of chemokines molecules on both parental and modified cells. Finally, apoptosis assays revealed the same rate of apoptosis for both T cell and modified T cells confirming that: 1-modified-T cells were protected by our "protecting" vectors. 2-transduction with the IT vector does not confer a survival advantage to the modified cells, and thus supports the safety of this approach. Lastly we showed that IT constitutively produced by T cells (under a CMV promoter) could kill $CD22^+$ tumors, in these test T cells supernatants were use on K562 and LCL cultures.
In year 3, we have confirmed all these data using tumor–specific T cells lines containing both $CD4^+$ and $CD8^+$ cells (instead of OKT-3 T cells that are not specific to the tumor). We have also shown that IT released by $CD4^+$ cells are not affecting the killing function of $CD8^+$ cells and that both killing mechanisms are eliminating $CD22^+$ tumors cells.

Figure 1: Analysis of apoptosis rate of IT producing T cells. The phenotype of parental and IT modified tumor-specific T cells was compared, Unmodified T cells, T cells transduced with LN-CMV-IT, $CD4^+$ T cells and $CD4^+$ T cells transduced with LN-CD40L-IT were activated with anti-CD3 and anti-CD28 overnight and then stained with Annexin-V FITC and PI followed by flow cytometry analysis. The level of apoptosis in resting and activated parental T cells and IT-modified T cells was essentially identical.

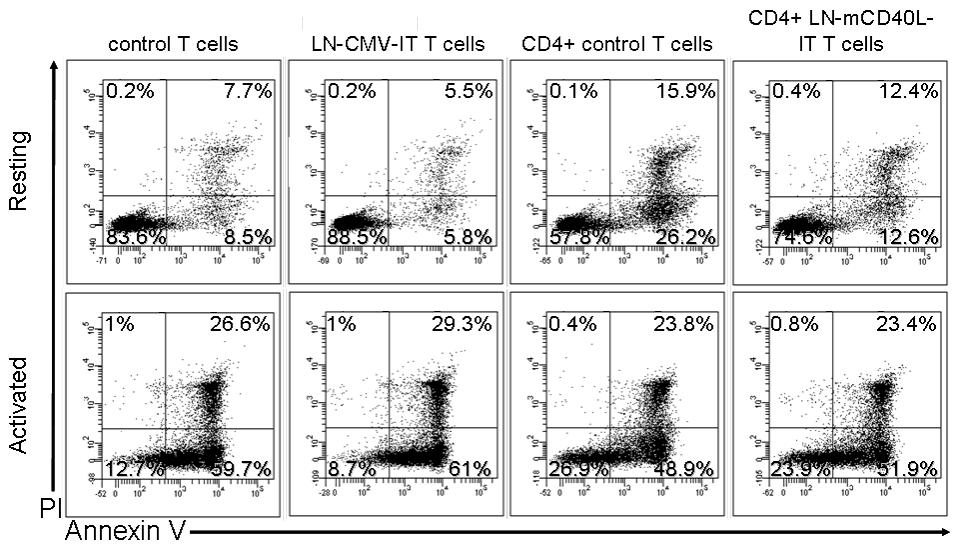


Figure 2: Cytotoxic activity of the T cells was unimpaired by IT expression. Control and LN-CMV-IT T cells were activated overnight with CD3/CD28, then incubated with CSFE labeled $CD22^+$ LCL target cells at an effector to target ratio of 5:1. After 4 hours, the percentage of LCL lysis was analyzed by flow cytometry using PI as a marker of dead cells. We found that the cytotoxic activity of the T cells was unimpaired by IT expression. Cytotoxicity was not significantly different between modified and unmodified populations

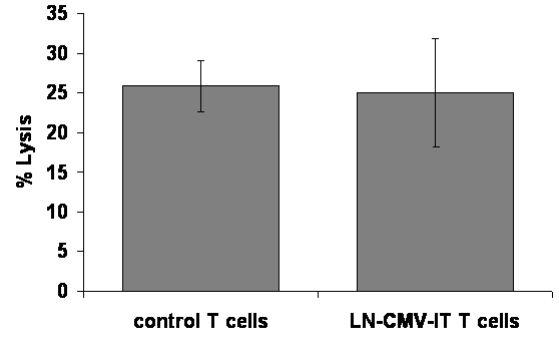


Figure 3: CD40L-regulated IT specifically kills CD22$^+$ tumors. T cells transduced with LN-CMV-IT and CD4$^+$ T cells transduced with LN-CD40L-IT were activated with anti-CD3 and anti-CD28 antibodies overnight and their supernatants used to treat CD22$^-$ Jurkat and K562 and CD22$^+$ LCL. On the sixth day the cell death was evaluated using Trypan blue staining. Six days after treatment with IT supernatants, 35% of CD22$^+$ target cells had been killed by supernatants from both activated CD4$^+$ cell population. Control CD22$^-$ target cells were unaffected by IT supernatants, confirming the specificity of the IT killing.

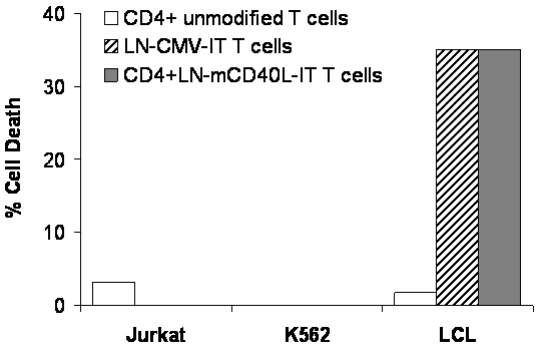


Figure 4: CD8$^+$ T cell killing is not impaired by IT. To ensure that the presence of IT will not interfere with CD8$^+$ T cell killing via these mechanisms, we used cytotoxic T lymphocytes (CTLs) specific for the autologous target LCLs, rather than the non-specific T cells. LCL-specific CTLs (containing non-transduced CD4$^+$ and CD8$^+$ cells) and target CD22$^+$ LCL were mixed and resuspended in supernatant from 293T cells transfected with a control GFP plasmid or a LN-CMV-IT plasmid, and incubated at 37°C. After 4 hours, cell death was evaluated using flow cytometry. Over this short assay period, most killing observed would be attributable to conventional CD8$^+$ T cell mechanisms, since the activity of the IT is not manifest until 24 hrs or more have elapsed. As expected, the cytotoxic activity of CTLs combined with the IT supernatant was not impaired and was only slightly greater than CTLs alone, so although the presence of IT in this short term assay does not contribute to the target cell killing, neither does it hinder the effect of CTLs.

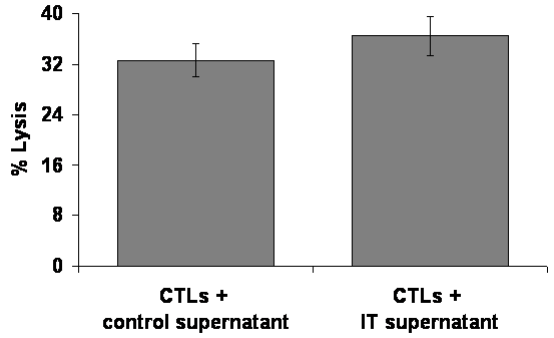
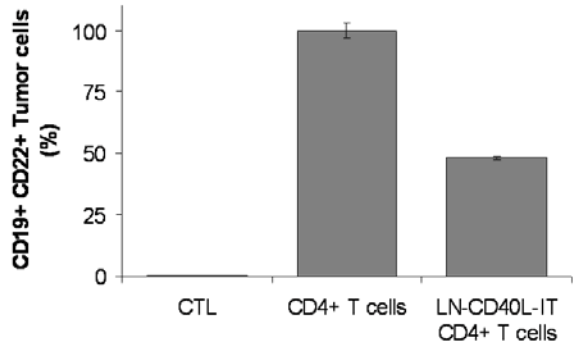


Figure 5: Both IT-modified CD4$^+$ and tumor–specific CD8$^+$ kill CD22$^+$ tumors. To address the effect of IT produced from CD4$^+$ T cells activated by autologous CD22$^+$ tumor cells, autologous tumor-specific CD4$^+$ T cells were sorted from our EBV- specific CTL lines and transduced with the LN-CD40L-IT vector. Unmodified CTLs (Containing 80% of CD8$^+$ cells and 20% of CD4$^+$ cells), CD4$^+$ T cells, and CD4$^+$ T cells transduced with LN-CD40L-IT, were mixed with CD22$^+$ LCL at a ratio of 4: 1. Following 5 days co-culture, the percentage of remaining LCLs was evaluated using CD19-specific flow cytometry. Results showed that the total target cell numbers were unaffected when cultured with unmodified CD4 cells but that CTLs were able to completely kill the LCLs. Results expressed as the percent of target cells relative to what was obtained with unmodified CD4$^+$ T cell controls revealed that the IT modified CD4$^+$ T cells were able to kill over half the CD22$^+$ target cells.



In conclusion our data revealed that compared to recombinant IT or cell-mediated tumor eradication, our unique IT producing mEF-2/T cells (and tumor-specific CTLs) will locally deliver a high amount of IT, while still killing via granzyme, perforin and cytokine release, thus providing a safe and potent therapeutic approach to specifically and efficiently eradicate CD22$^+$ B-cell malignancy.

KEY RESEARCH ACCOMPLISHMENTS.

- Generation of stable CD4$^+$ and T cell lines cells that express a mutated elongation factor resistant to PEA toxin and the IT gene regulated by CMV or CD40L promoter.
- Characterization of the immunotoxin-modified T cell lines.

- Evidence that the phenotype and function of the immunotoxin –modified T cells, $CD8^+$ and $CD4^+$ are not altered by the IT genetic manipulation.

- Evidence that immunotoxin-modified T cells and $CD4^+$ produce immunotoxins.

REPORTABLE OUTCOMES.
-Development of cell lines:
(CD40L Promoter) IT producing $CD4^+$ and T cell lines
(CMV Promoter) IT producing $CD4^+$ and T cell lines

CONCLUSIONS.

Most tumors (including aggressive and metastatic tumors) are resistant to classical treatments. Cancer patients often relapse after responding well to single therapies. These situations occur because tumors develop escape mechanisms that allow them to resist the host immune response and to single therapy but also to evolve into more aggressive tumors.

Our project uses a unique approach of double killing mechanism targeting two different tumor molecules and using different killing mechanisms. This would prevent tumors escape and thus significantly improve and accelerate their eradication.
The majority of B-malignant cells express CD22 antigen, therefore a CD22-based IT could be of great importance in the treatment of many B-cell leukemias and lymphomas. In this project we target CD22 and EBV on $CD22^+$ B-malignancies.

Our results showed that our IT-engineered T cells could be specifically activated by autologous tumors cells to produce IT. The IT and the perforins specifically produced by these T cells following activation efficiently kill the tumor. Importantly this killing is superior to the killing of T cell alone or to IT alone. Based on these promising results, we have applied for a R01-NIH award to verify in vivo the efficacy and feasibility of this approach in a mice model.

Salaries supported:
PI: Yotnda Patricia
POST-DOC: Anna Swanson and Danli Wu,

REPORTABLE OUTCOMES:
- Manuscripts: in preparation see appendices.

-Presentation at meetings:
2007 13th International Congress of Immunology
2009 DOD annual meeting
2010 International meeting of immunology (August 2010)

-Funding applied for based on work supported by this award:
R01–NCI/NIH pending (resubmission)

APPENDICES: Manuscript (draft) attached. Dr Y otnda will n otify USAMRMC w hen th e manuscript is published so it can be credited as an outcome from this grant.

Delivery of tumor-targeted immunotoxin by tumor-specific T cells.

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Introduction.

Conventional treatment for lymphoma is associated with a high frequency of treatment-related toxicity and a lower than desired cure rate. The development of effective antibody and, more recently, cell based immune therapies directed against clonal or lineage-restricted B cell antigens has therefore been of considerable value[1-5], and a monoclonal antibody to CD20 (Rituximab) is now used in front line therapies for this disease[6-11].

Monoclonal antibodies act via direct cellular toxicity, or by complement fixation and antibody-dependent cell mediated cytotoxicity. Many target cells, particularly those expressing low concentrations of the antigen, are damaged only transiently, if at all by these effector mechanisms[10]. Researchers have sought to enhance the effector function of monoclonal antibodies by coupling toxic moieties to the targeting portion of the immunoglobulin[12-15], so that the toxin is internalized once the antibody binds to the cell surface[16-22]. For example, CD22-*Pseudomonas exotoxin A* (CD22-PEA) contains the translocating and ADP-ribosylating domains of PEA[23], but the native cell-binding portion is replaced with a CD22 moiety that directs targeting to B lymphocytes[24]. CD22-PEA has been tested in Phase I and II trials in B-cell malignancies, but tumor responses were accompanied by an unfavorable toxicity profile in the liver and kidneys[25,26]. Although the IT approach has shown promise in animal models, toxicity issues have limited its clinical application[27-32]. Only a small proportion of the drug reaches target cells after systemic injection, meaning that relatively high doses of IT are required to induce a significant biologic effect. Consequently, the increased killing of target cells is partly offset by increased toxicity. T lymphocyte therapies have also been used to treat lymphoma. Cytotoxic T cells (CTL) recognizing tumor-specific immunoglobulin idiotypes or isotypes have been tested[5], and therapeutic administration of CTLs specific for the EBV-specific antigens expressed by a proportion of Hodgkin and non-Hodgkin lymphomas is safe and produces a high rate of complete and sustained remission in these diseases[33]. Adoptively transferred EBV-CTLs expand and survive long term in patients with lymphoma, and also traffic to tumor sites where they induce tumor regression.

We now describe a combination T cell-immunotoxin therapeutic that exploits the beneficial properties of each component, and delivers both a cytotoxic T cell and a high concentration of immunotoxin to tumor cells. When a T cell encounters its cognate antigen, a cascade of events ensues, including the transitory upregulation of costimulatory molecules. For example, expression of CD40L, which is largely restricted to CD4 T cells is first noted within one hour of antigen contact, peaks at eight hours and is undetectable by 48 hours[34]. Placing the IT under the control of the tightly regulated CD40L promoter ensures the IT is released only when the T cell engages with its malignant target cell, thereby minimizing the total body exposure to the agent. These CD22-PEA-engineered, cytotoxic T lymphocytes have increased tumor-specific killing activity compared to non-modified CTLs.

Material and Methods.

Cells.

293T (human embryonic cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc, Manassas, VA). Jurkat (T leukemia), EBV transformed B lymphoblastoid cell lines (LCL) and K562 (erythroleukamia) were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Mediatech Inc). T cells were cultured in RPMI-1640, supplemented with human recombinant (hr)IL-2 at 50 U/ml (proleukin; Chiron, Emeryville, CA). All media were

supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml Amphotericin B and 2 mM L-glutamine, (all from Mediatech) and 10 or 20% fetal calf serum (Hyclone, Logan, UT). Cells were maintained at 37°C, 5% CO_2 in a humidified incubator.

Generation of T cells.
T cells were prepared by stimulating peripheral blood mononuclear cells with antibodies against CD3 and CD28 (BD Pharmingen, Franklin Lakes, NJ). 24 well culture plates were coated with 0.5μg/ml of each antibody for 2-4 hours at 37°C. Cells were added at $0.5x10^6$ cell /ml in 2ml medium per well and cultured for up to 72 hours. Cells were fed with media containing hrIL-2 (50 IU/ml) twice weekly. CTLs were prepared by stimulating peripheral blood mononuclear cells with autologous EBV-transformed LCLs. Peripheral blood mononuclear cells (2×10^6) were cocultured with 5×10^4 gamma-irradiated (40 Gy) autologous LCLs per well in a 24-well plate. On day 10, the responder cells were restimulated with irradiated (40 Gy) LCLs at a responder-to-stimulator ratio of 4:1. From day 14, hrIL-2 (50 IU/ml) was added twice weekly and the cells restimulated with irradiated (40 Gy) autologous LCLs at a responder-to-stimulator ratio of 4:1 once a week.

Recombinant immunotoxin.
BL22 (HA22PE38, (HA22)), LMB-9 (B3 (sFv)-PE38) and the single chain CD22-PEA were kind gift from Dr I. Pastan. The development and production of the disulfide-linked recombinant immunotoxin, BL22 (HA22PE38 was previously described[23] and contains the mutated anti-CD22 engineered to have an increased affinity for CD22[35]. Recombinant immunotoxins were stored in 0.9% saline containing 0.2% human serum albumin at -80°C and thawed at room temperature immediately before use.

Vector construction.
The cassette encoding the CD22 single chain antibody fused in-frame to the truncated PEA toxin was linked to the leader peptide of human IL-15. This fragment was cloned into the PRc/CMV plasmid and the expression cassette subcloned into a lentivirus vector under the regulation of a CMV promoter to create LN-CMV-CD22-IT (Figure 2a). To construct an activation-inducible LN-CD40L-CD22-IT vector, the CMV was replaced with the CD40L promoter. To protect virus producer cells from the IT, the LN-CMV-mEF2-IRES-GFP vector was constructed using a CMV promoter to drive expression of a mutant elongation factor 2 (mEF2) gene. To facilitate selection, green fluorescent protein was inserted following an encephalomyocarditis virus internal ribosome entry site (IRES).

Vector production and transduction.
The self-inactivating lentiviral vectors were prepared using 293T cells. The cells were transfected with the lentiviral constructs and the envelope and accessory plasmids (pMD.G encoding VSV-G and pCMVΔR8.9). The virus preparations were filtered and concentrated by ultra centrifugation (1 hour at 27000 rpm and 4^0C), and cells were spinfected at 1100g for 60 minutes in the presence of the virus and Polybrene (8μg/ml, Sigma St Louis, MO).

Transduction level.
We confirmed transduction of the T cells using real time PCR. DNA was extracted from approximately $5x10^5$ cells using the QIAamp DNA Mini Kit (Qiagen), and eluted in 200μl.

Primers were designed using the Primer Express 2.0 software to amplify a region of the PEA gene: forward 5'-CTATGTGTTCGTCGGCTACCAC-3'and reverse 5'-AAGACGATGCTTTGCGCC-3'. For the detection of the albumin gene, the following primers were used: forward 5'-TGAAACATACGTTCCCAAAGAGTTT-3' and reverse 5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3' [36]. For the PCR, 12.5µl iTaq Sybr Green Supermix (BioRad, Hercules, CA), 200mM each primer, and 10µl template DNA, with nuclease free water to a total volume of 25µl. Cycling conditions were 50°C for 2 minutes, 95°C for 3 minutes, and then 40 cycles of 95°C for 15 second and 60°C for 30 seconds. No template reactions were included with each experiment as a negative control, and each sample was performed in duplicate. Copy number was quantified using standard curves with 10-fold serial dilutions of plasmid (PEA) or cellular DNA (albumin). Vector integrations were calculated by normalizing the number of PEA molecules to the number of cells, as quantified by the copy number of albumin molecules detected on the same sample, and are expressed as vector copies per million cells.

Proliferation assay.
Unmodified or transduced cells were seeded at $8x10^4$ cells per well in a 96-well plate and cultured with 20U/ml hrIL-2 (resting), 50U/ml hrIL-2, or OKT3/CD28+20U hrIL-2/ml (plate coated with 0.5µg/ml each antibody for 2 hrs) for 3 days at 37°C in 5% CO_2. The level of proliferation was then assessed using an ATP assay (CellTiter-Glo G7570; Promega, Madison, WI).

Phenotyping.
Cell-surface phenotype was investigated using the following monoclonal antibodies: CD3, CD4, CD8, CD19, CCR7, CXCR4, CD31, and CD62L (eBioscience, San Diego, CA). Cells were analyzed with a FACSAria flow cytometer (BD Biosciences).

Apoptosis assay.
T cells were stimulated overnight with 3µg/ml PHA and 20U/ml hrIL-2 and analyzed using an eBioscience apoptosis detection kit (Becton Dickinson Bioscience, Mountain view, CA). Briefly, the cells were stained with Annexin-V and 7-Aminoactinomycin D (7AAD) or propidium iodide (PI) according to the manufacturer's instructions and analyzed by flow cytometry using a FACSAria.

Migration assay.
T cell migration in response to cytokines was assayed in 24 well plate transwell chambers with a 3µm pore polycarbonate membrane (Corning Inc, Corning, NY). Fresh or conditioned media was added to the lower chamber of the transwells, and $5x10^5$ unmodified or transduced cells in fresh media were added to the upper chamber. Conditioned media was collected from human macrophages which had been cultured overnight in 50ng/ml LPS, as activated macrophages secrete the chemokine SDF-1, the ligand for CXCR4. The plates were incubated for 4.5 h at 37°C in 5% CO_2. Migrating cells were collected in the lower chamber and counted using trypan blue.

Supernatant Cytotoxicity.
The lytic activity of the immunotoxin was evaluated using culture supernatants from IT-modified cells. $CD22^+$ LCL and $CD22^-$ Jurkat or K562 cells were cultured with supernatants from unmodified cells, 293 cells transfected with LN-CMV-IT, T cells transduced with LN-CMV-IT, or activated T cells transduced with LN-CD40L-IT and cultured at 37°C in 5% CO_2. Treated cells and control cells were cultured in 96-well round bottom culture plates for 3 days when using 293 supernatants and for 7 days when using T cell supernatant (supernatant were renewed on the third and fifth days). Cytotoxicity was assessed at using trypan blue or an ATP assay (Promega).

Cytotoxicity assay.
T cell cytotoxic activity was evaluated in a flow cytometry based assay. Target cells were stained with 5μM CSFE (Molecular Probes Invitrogen, Carlsbad, CA), before plating with effector cells. After incubation at 37°C for 4 hours, PI was added to the mixture, and the percentage of dead target cells assessed by flow cytometry. Spontaneous death was calculated by incubating target cells without effector cells. The percentage cytolytic activity was calculated as (CSFE+ve PI+ve cells)/(CSFE+ve PI+ve + CSFE+ve PI-ve cells)x100, with spontaneous death subtracted as background. An effector:target ratio of 5:1 was used, as preliminary tests with T cells showed there to be a sufficient distinction between lysis levels in targets and controls (data not shown).

Co-Culture Assay.
The ability of $CD4^+$ T cells transduced with LN-mCD40L-IT to kill target cells using the IT was assessed using a co-culture assay. Unmodified CTLs, $CD4^+$ T cells, and $CD4^+$ T cells transduced with LN-CD40L-IT, were mixed with $CD22^+$ LCL at a ratio of 4:1 and cultured in 40U/ml hrIL-2 at 37°C in 5% CO_2 for 5 days. To evaluate the level of cytotoxicity, cells were harvested, stained with anti-CD3 and anti-CD19 and analyzed by flow cytometry to investigate the number of $CD19^+$ target cells remaining alive. The total number of $CD19^+$ target cells remaining following incubation with normal unmodified $CD4^+$ cells is used as control for comparison of target cell killing by the IT.

Statistical analysis.
For statistical analysis, a student t-test was used. For each test, P-values of <0.05 were considered statistically significant.

Results.

The CD22-PEA immunotoxin specifically kills B-lymphoma cells.
B cell malignancies express high levels of $CD22$[37,38] as do the LCLs and B cell lines (Daudi, BJAB Ramos and Raji) used in the current study (Figure 1a). To determine the effects of CD22-toxin fusion proteins on these cells we first used an apoptosis assay. $CD22^+$ leukemic B cells (Daudi) and $CD22^-$ leukemic T cells (Jurkat) cells were treated with recombinant CD22-toxin (HA22, 5ng/ml). Untreated $CD22^+$ Daudi and $CD22^-$ Jurkat cells were used as controls. As immunotoxin control, we used the irrelevant immunotoxin LMB-9 (B3 (sFv)-PE38, 5ng/ml); a disulfide-linked immunotoxin specific for the $Lewis^Y$ antigen, which is absent from lymphocytes. Cell apoptosis was evaluated for each group using Annexin and 7AAD. Our results showed that 41.8% of $CD22^+$ Daudi cells were apoptotic in the presence of the recombinant CD22-

immunotoxin versus 21.8% of non-treated control cells. The anticipated level of attrition for these cells (Figure 1b). More cells were double positive for 7AAD and Annexin-V in CD22-PEA-treated cultures (35.9%) versus the irrelevant control LMB-9 immunotoxin treated cultures (6.8%). Conversely, $CD22^-$ Jurkat cells showed similar levels of apoptosis irrespective of whether they were untreated or treated with CD22-PEA or control LMB-9 immunotoxin, indicating that treatment with the CD22-PEA resulted in the death of $CD22^+$ but not $CD22^-$ cells.

Construction and validation of the CD22-toxin vectors.

To create a system in which CD22-PEA is produced only when the effector T cell receptor is engaged following an encounter with its malignant target, we prepared a CD22-PEA plasmid in which we substituted the CD40L- for the CMV-promoter (see materials and methods). To confirm that CD40L-promoted CD22-PEA would be produced only after T cell activation, we also prepared a firefly luciferase construct driven by the same CD40L promoter. Jurkat T cells were transfected with the CD40L-driven construct and then activated with 5 ng/ml PMA and 1μM ionomycin. Cells were collected and lysed, and the lysate analyzed for bioluminescent signal. This revealed that the promoter induced signal only following T cell activation of transduced cells (Figure 2b).

Protection of immunotoxin producing cells.

If immunotoxin is not to kill the cells that are producing it they must be protected from its adverse effects. The sole cellular target of PEA is the elongation factor-2 (EF-2), irreversible inactivation of which by PEA blocks protein synthesis, and causes cell death. EF-2 can be mutated to reduce the affinity to toxin and prevent binding, without loss of function[39]. We therefore constructed a lentiviral vector containing this mutant EF-2 (mEF-2) under the control of the CMV promoter (Figure 2a). In addition to protecting T cells producing the immunotoxin, this vector should also allows the 293T cells we use as viral vector producer cells to be resistant against any PEA protein made during vector synthesis.

To validate the protective effect of mEF-2, 293 T cells were transfected with the LN-mEF2 vector approximately 8 hours prior to transfection with a GFP expression vector and the LN-CMV-IT (CD22-PEA) vector (Figure 3a). Control samples consisted of 293T cells transfected with GFP vector only or with GFP and LN-CMV-IT vectors but without LN-mEF2. At 48 hours, cells were assessed by fluorescent microscopy. 293T cells expressing the GFP vector alone had high levels of GFP expression, but cells transduced with GFP and LN-CMV-IT produced only a weak signal. As hoped, however, prior transfection with the LN-mFE2 vector protected expression in GFP and IT treated cells.

To confirm CD22-toxin production by 293T cells, we performed western blot and immunoprecipitation analyses (IP). 293T cells were modified with the mEF-2 vector either 24 hours prior to, or on the day of transfection with GFP and IT vectors. Control 293T cells were transfected with GFP alone or GFP and LN-CMV-IT. Cells were collected 48 hours after transfection, and assayed by Western blot (Figure 3b). We confirmed that 293T cells produced most IT if they were protected by mEF-2 before expression of the IT. Immunoprecipitation performed with the supernatant from protected 293T cells modified with IT vector (Figure 3c) confirmed that the IT is secreted by IT producer cells.

To assess the relative cytotoxic activity of IT from protected and non-protected IT producer cells, we collected supernatant 48 hours following IT vector transfection, and added this to $CD22^+$ (LCL) and $CD22^-$ (Jurkat) cells lines. Supernatant harvested from GFP transfected 293T cells was used as control. After three days of culture cytotoxicity was assessed using an ATP assay, which measures the cells that are alive, with data normalized to GFP controls. Supernatant from IT transfected producer cells was able kill 85.6% of $CD22^+$ LCL cells while the $CD22^-$ Jurkat cells were minimally affected (30% cell death) (Figure 3d). Supernatant from non-protected 293T cells killed 25% of $CD22^+$LCL with no toxic effect (0% cell death) on $CD22^-$ Jurkat cells. All further virus production used 293T cells protected with a mEF-2 vector containing GFP for selection (Figure 2a).

Expression of an immunotoxin did not alter T cell characteristics.

T cells were armed with the CD22-targeted IT by transduction with vectors encoding the IT under the control of either a CMV or CD40L promoter (Figure 2a). T cells were not protected with mEF2 so as to more clearly measure the effects of IT production on the therapeutic cells. However, such protection could be extended to these cells for *in vivo* studies, where the long term survival of the cells would be of import. The transduction efficiency of the T cells with the IT constructs was assessed using real time PCR with primers designed to amplify a sequence within the PEA gene. All cells used in these experiments had a transduction level of at least $1x10^6$ vector copies/million cells (data not shown).

To discover whether IT expression alters T cell characteristics, we compared the phenotype of modified and unmodified T cells using CD3, CD4, and CD8 antibodies. Our FACS analysis revealed that IT expressing cells had unaltered proportions of $CD3^+$, $CD4^+$, or $CD8^+$ cells (Figure 4a). We next assessed whether IT-modified T cells maintained their expression of the chemokine receptors (CCR7, CXCR4) and adhesion molecules (CD31 and CD62L). The FACS data in Figure 4b shows that modification of T cells with the IT did not change expression levels of these T cells at rest or following activation, with the exception of a lower expression of CD62L. To demonstrate that these IT-modified T cells were also able to responding to chemokines whose receptors they expressed, we incubated both parental and IT producing T cells in transwell inserts placed in receiver wells containing either fresh or conditioned (SDF-1 enriched) media. After incubation at 37°C, fewer than 15% of IT-modified T cells in control wells migrated, compared to 35% of IT-T cells incubated with SDF-1 enriched media (Figure 4c); 49% of unmodified T cells migrated in the presence of SDF-1, while 14% migrated in fresh media.

We next investigated if IT-engineered T cells retained their response to activation stimuli. Parental and IT-modified T cells were exposed to hrIL-2 (50U/ml) or CD3/CD28 antibodies (1mg/ml) or cultured unstimulated. In an ATP proliferation assay after three days culture, both parental and transduced T cells responded equally to CD3/CD28 and to 50UI/ml of hrIL-2 (Figure 4d). Both parental and modified T cells were then cultured in the presence of recombinant human hrIL-2, and then starved by IL-2 withdrawal. We obtained viable cell counts using Trypan blue, and found that IT expression did not remove T cells dependence on IL-2 for continued growth and survival (Figure 4e). Hence IT producing T cells are not transformed to growth factor independence.

The level of apoptosis in resting and activated parental T cells and IT-modified T cells was essentially identical (Fig 4f). This applied to both $CD4^+$ and $CD8^+$ subsets (4f). Although apoptosis of activated T cells is higher than of resting T cells; this apoptosis rate is unaffected by IT expression. Finally, we found that the cytotoxic activity of the T cells was unimpaired by IT expression. As shown in Fig 4g, incubation of T cells with CSFE labeled LCL target cells showed that at 4hrs cytotoxicity was not significantly different between modified and unmodified populations (P=0.885). This is an autologous system, with both T cells and target LCL derived from the same donor. T cells were not expanded using a specific antigen.

Cytotoxicity of the immunotoxin

To test for production of active immunotoxin from IT modified T cells we collected supernatants from untransduced and transduced T cells following activation with CD3 and CD28. Cells were a mixed population of $CD8^+$ and $CD4^+$ cells modified with LN-CMV-IT or sorted $CD4^+$ cells modified with LN-CD40L-IT. Supernatant was then added to $CD22^+$ LCL, and to $CD22^-$ Jurkat and K562 cells. Cell death was evaluated by trypan blue counts and the data normalized to the untransduced mixed population. Figure 5a shows that six days after treatment with IT supernatants, 35% of $CD22^+$ target cells had been killed by supernatants from both activated $CD4^+$ cell population. Control $CD22^-$ target cells were unaffected by IT supernatants, confirming the specificity of the IT killing. These cytotoxic effects were mediated by the IT alone, and not via the release of any cellular factors (granzyme, perforin, TNF), as lysis was only 3% when we use supernatant from activated but unmodified T cells (Figure 5a).

An important method by which T cells kill targets involves the release of granzymes and perforins. To ensure that the presence of IT will not interfere with $CD8^+$ T cell killing via these mechanisms, we used cytotoxic T lymphocytes (CTLs) specific for the autologous target LCLs, rather than the non-specific T cells. We obtained supernatant from 293T producer cells modified with the LN-CMV-IT vector. CTL cells (containing $CD4^+$ and $CD8^+$ cells) mixed to autologous LCL ($CD19^+$, $CD22^+$) targets were resuspended in fresh control or IT supernatant and incubated for 4 hours at 37°C. Cytotoxic activity was evaluated by flow cytometry. Over this short assay period, most killing observed would be attributable to conventional $CD8^+$ T cell mechanisms, since the activity of the IT is not manifest until 24 hrs or more have elapsed. As expected, the cytotoxic activity of CTLs combined with the IT supernatant was not impaired and was only slightly greater than CTLs alone (Figure 5b), so although the presence of IT in this short term assay does not contribute to the target cell killing, neither does it hinder the effect of CTLs.

To address the effect of IT produced from $CD4^+$ T cells activated by autologous $CD22^+$ tumor cells, autologous tumor-specific $CD4^+$ T cells were sorted from our EBV- specific CTL lines and transduced with the LN-CD40L-IT vector then cultured with target LCLs at an effector to target ratio of 4:1. After 5 days co-culture, the percentage of remaining LCLs was evaluated by flow cytometry using CD3- and CD19-antibodies (Figure 5c). Controls consisted of unmodified CTLs which were >95% $CD8^+$ (positive controls), and of sorted but unmodified $CD4^+$ T cells (negative control). Results showed that the total target cell numbers were unaffected when cultured with unmodified CD4 cells but that CTLs were able to completely kill the LCLs. Results are thus expressed as the percent of target cells relative to what was obtained with unmodified $CD4^+$ T cell controls and revealed that the IT modified $CD4^+$ T cells were able to kill over half the $CD22^+$ target cells.

Discussion.

The majority of B-malignant cells express CD22 antigen, therefore a CD22-based IT could be of great importance in the treatment of many B-cell leukemias and lymphomas. Most ITs are currently injected intravenously, however, this route of IT delivery has numerous limitations such as the systemic IT dilution, their high toxicity to normal tissues when used at high doses (vascular leak syndromes, thrombocytopenia, and liver damage), their short serum half-life, their rapid clearance from the circulation due to their immunogenicity, and their inability to reach poorly or non-vascularized tumors. Considering that non-vascularized tumors are often also aggressive and metastatic tumors, this limitation is a major drawback. Effective therapies will depend on the efficient killing of these non-vascularized tumors. Finally, recombinant-IT also raises problems associated with their long term storage. Thus, for protocol requiring multiples dose of injections there is a need to guarantee that an identical unimpaired recombinant IT can later be injected several times. Recombinant CD22-PEA has already been described as killing B-malignant cells such as hairy leukemia, and encouraging results have been obtained in the clinical setting. In this study, we use a modified CD22-PEA immunotoxin that has been modified to be less immunogenic. We propose to T cells to deliver this IT directly to tumor cells thereby improving efficacy and safety[40,41]. To increase the specificity and safety of the delivery and to have an additional killing mechanism we used tumor-specific T cells. These cells have the ability to migrate, cross vessel walls and tissue barriers, and infiltrate even non-vascularized tumors. Since the IT produced by T cells will be locally released at the tumor site itself, the problems encountered with recombinant IT and cited above are no longer a limitation. Local delivery by engineered T cells will prevent IT toxicity to normal tissue, systemic IT dilution, and the induction of an immune response directed against the IT moiety, thus allowing repeated infusions. It will also prevent the access of the IT to neutralizing antibodies and would allow the treatment of patients previously infused with recombinant IT. Because our T cells contain both CD8 cells specific for a tumor antigen different than CD22 and tumor-specific CD4 cells producing IT targeting CD22 antigenc, our strategy targets two different tumor antigens, which is crucial to circumvent tumor escape (by downregulation of a single antigen). Finally, T cells home to the primary tumor but also to metastases and therefore will efficiently distribute the IT to completely eradicate the malignant cells. Other safety and efficacy problems raised at different steps of the production of recombinant IT proteins from insoluble bacterial inclusion bodies and concerning the proper folding of the fusion protein are now inexistent since the IT is produced by mammalian human cells. Our system also relies on the activation dependent CD40L promoter regulating the release of IT by T cells, an additional level of safety.

Our data showed that IT (CD22-PEA immunotoxin) can be produced by engineered primary human T cells. We showed that the IT producing cells kept their CD3, CD4 and CD8 phenotype. The expression of chemokine receptors and adhesion molecules was also largely unchanged on their cell surface. The level of CD62L was slightly decreased on activated modified T cells. However, IT producing T cells retained their ability to migrate in response to SDF-1. We observed a minor reduction of migration in our assay that is not due to a change in the expression of CXCR4 (the ligand of SDF-1) nor to a change of T cells size (physical limitation). We have used T cells modified with the IT under regulation of the constitutive promoter (LN-CMV-IT) to mimic extreme conditions of high and constitutive production of IT for these migration studies and we do not anticipate the expression of the IT under the inducible CD40L promoter to affect

migration to such an extent. Additionally, the IT will be produced by T cells in the tumor where migration is no longer needed.

We also investigated if the manipulation of hematopoietic T cells with an IT encoding lentivirus could be a safety issue and found that IT-modified T cells proliferate and undergo apoptosis to the same extent parental T cells do. Furthermore, we found that the proliferation of IT producing T cells is still dependent on hrIL-2 and T cell receptor (TCR) signaling. Altogether, these results showed that our modified T cells were not transformed (malignant) following genetic modification with our IT and mutated elongation factor. During the 4 hour cytotoxicity test, IT is produced by IT-$CD4^+$ T cells. However, this assay investigates almost exclusively the $CD8^+$ T cell-mediated killing (granzyme/perforin) since PEA-mediated death requires a longer period of incubation. Indeed, PEA inhibits elongation factor function and kills LCL target cells by preventing the synthesis of new proteins, thus its lytic effect is delayed. Results validated that $CD8^+$ T cell cytotoxic function was unchanged in presence of IT released by $CD4^+$ T cells as both control and T cell line containing IT producing T cells have equal lytic activity. This finding strongly suggests that the combination/addition of both $CD8^+$ cells and IT cytotoxic effects would provide a greater therapeutic effect compared to each therapeutic approach considered individually. Over a five day co-culture assay, $CD4^+$ T cells transduced with the LN-CD40L-IT vector were able to kill over half the target population, further supporting this idea.

Using a luciferase gene reporter assay, we demonstrated that CD40L promoter is “turned on” only after T cell activation. Our cytotoxicity test using CD40L-IT modified $CD4^+$ T cells (sorted or combined with $CD8^+$ T cells) confirmed that the IT is produced following T cell activation and revealed that IT efficiently kill $CD22^+$ target cells. These data validate the biological cytolytic activity of our IT and show that the inducible release of the immunotoxin is efficiently regulated by CD40L promoter. Our findings clearly demonstrated the increased safety of our system. It is our intention to use the IT producing $CD4^+$ cells in combination with tumor-specific $CD8^+$ cells. In physiological context, a cell-to-cell contact will occurs and as a result, the total cytotoxic effect of T cells (containing both $CD4^+$ and $CD8^+$ cells) will be higher given that $CD8^+$ cells will also participate to the eradication of tumor cells via granzyme/perforin killing. In support of this, we found in a 4 hour assay that IT did not interfere with $CD8^+$ cell killing activity.

In conclusion our studies revealed that compared to recombinant IT or cell-mediated tumor eradication, our unique IT producing mEF-2/T cells (and tumor-specific CTLs) will locally deliver a high amount of IT, while still killing via granzyme, perforin and cytokine release, thus providing a safe and potent therapeutic approach to specifically and efficiently eradicate $CD22^+$ B-cell malignancy.

Figure legends.

Figure 1: Targeting $CD22^+$-malignant cells with a CD22-immunotoxin.
a) B-malignant cell lines were stained with a monoclonal anti-CD22 and isotype control antibodies and analyzed by flow cytometry. A representative histogram is showed for an LCL, Daudi and BJAB cell line. b) $CD22^-$ Jurkat and $CD22^+$ Daudi cells (1 x 10^6 cells/ml) were incubated with recombinant immunotoxin CD22-PEA or control LMB-9 (5 ng/ml) for 3 days and analyzed for apoptosis using Annexin-V and 7AAD.

Figure 2: Construction of an immunotoxin vector where expression is induced by T cell activation.
a) Schematic of the vectors used in this study. The CD22 single chain antibody sequence was fused in-frame to the truncated PEA toxin, and linked to the leader peptide of human tyrosinase, with expression driven by either the constitutive CMV or inducible CD40L promoter. To protect producer cells from the effect of IT, a vector containing a mutant elongation factor 2 b) Jurkat cells were modified with a vector encoding the Luciferase gene under the control of the CD40L promoter. Transduced cells were activated with PMA and ionomycin and the luciferase signal was measured using a luminometer.

Figure 3: Protection of IT producing cells.
a) 293T cells were transfected with GFP alone, GFP+LN-CMV-IT, or with the mEF2 vector 7-24h hours prior to transfection with a GFP+ LN-CMV-IT. Images were taken at 48h post transfection using a fluorescent microscope. b) 293T cells were modified with GFP, mEF2+GFP+ LN-CMV-IT, mEF-2 24 hours prior to GFP+ LN-CMV-IT, or GFP+ LN-CMV-IT only. At 48 hours, IT production was assayed by Western blot. c) mEF2 modified 293T cells were transfected with GFP or IT vectors and the supernatant assayed at 48 hours by immunoprecipitation, and Western blot. d) $CD22^-$ Jurkat and $CD22^+$ LCL were cultured with supernatant from 293T cells transfected GFP, GFP+ LN-CMV-IT, or mEF2 24 hours prior to GFP+ LN-CMV-IT. After three days of incubation, the cytotoxicity was assessed using an ATP assay.

Figure 4: Analysis of phenotype of IT producing T cells.
a) the phenotype of parental and IT modified was compared, cells were stained with monoclonal antibodies specific for human CD3, CD4, and CD8 and analyzed by flow cytometry. b) Unmodified T cells and T cells transduced with LN-CMV-IT were activated with anti-CD3 and -CD28 then stained with antibodies specific for chemokine receptors CCR7 and CXCR4, and adhesion molecules CD31 and CD62L. Cells were analyzed by flow cytometry. c) Unmodified and T cells transduced with LN-CMV-IT were cultured in the presence of fresh or conditioned media, and their ability to migrate across a 3µm membrane assessed by trypan blue staining. d) Unmodified and T cells transduced with LN-CMV-IT were cultured in the presence of 20U/mL hrIL-2 (resting), 50U/mL hrIL-2, or CD3/CD28 antibodies and 20U/mL hrIL-2 for three days. Proliferation was assessed using an ATP assay. e) Unmodified and T cells transduced with LN-CMV-IT were cultured in the presence or absence of 50U/ml hrIL-2, and total live cells determined using trypan blue staining. f) Unmodified T cells, T cells transduced with LN-CMV-IT, $CD4^+$ T cells and $CD4^+$ T cells transduced with LN-CD40L-IT were activated with anti-CD3 and anti-CD28 overnight and then stained with Annexin-V FITC and PI followed by flow

cytometry analysis. g) Control and LN-CMV-IT T cells were activated overnight with CD3/CD28, then incubated with CSFE labeled $CD22^+$ LCL target cells at an effector to target ratio of 5:1. After 4 hours, the percentage of LCL lysis was analyzed by flow cytometry using PI as a marker of dead cells.

Figure 5: Cytotoxicity of T cells with IT.
a) T cells transduced with LN-CMV-IT and $CD4^+$ T cells transduced with LN-CD40L-IT were activated with anti-CD3 and anti-CD28 antibodies overnight and their supernatants used to treat $CD22^-$ Jurkat and K562 and $CD22^+$ LCL. On the sixth day the cell death was evaluated using Trypan blue staining. b) LCL-specific CTLs and target $CD22^+$ LCL were mixed and resuspended in supernatant from 293T cells transfected with a control GFP plasmid or a LN-CMV-IT plasmid, and incubated at 37°C. After 4 hours, cell death was evaluated using flow cytometry. c) Unmodified CTLs, $CD4^+$ T cells, and $CD4^+$ T cells transduced with LN-CD40L-IT, were mixed with $CD22^+$ LCL at a ratio of 4:1. Following 5 days co-culture, the percentage of remaining LCLs was evaluated using CD19-specific flow cytometry.

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Figure 1a

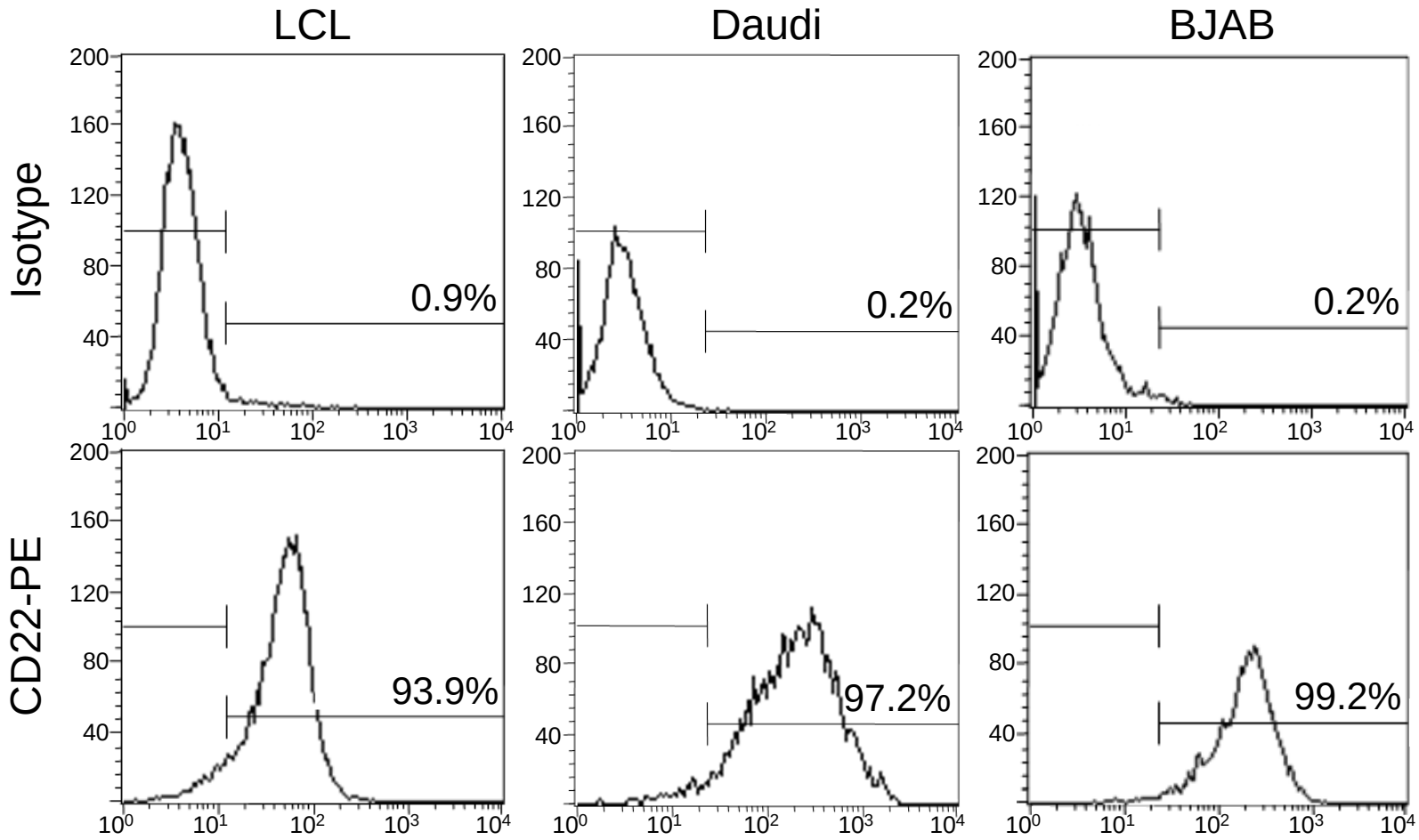


Figure 1b

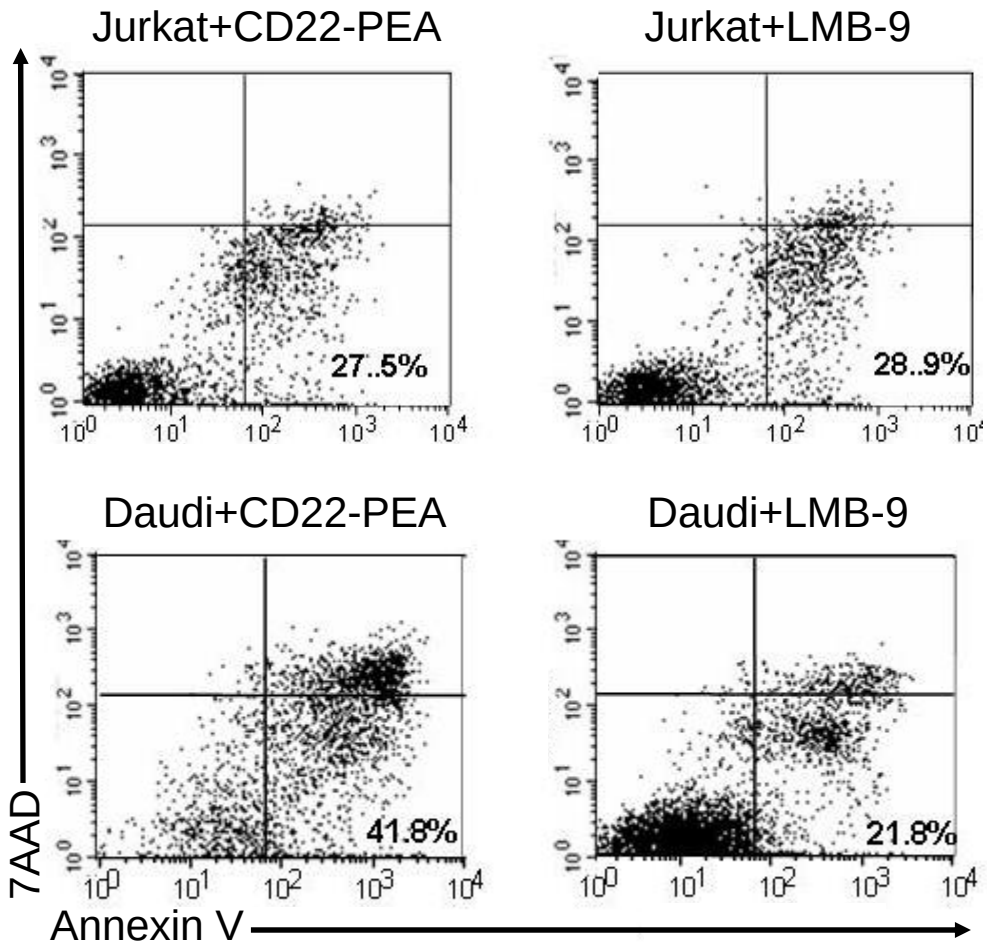

Jurkat+CD22-PEA
Jurkat+LMB-9
Daudi+CD22-PEA
Daudi+LMB-9
27..5%
28..9%
41.8%
21.8%
7AAD
Annexin V

Figure 2a

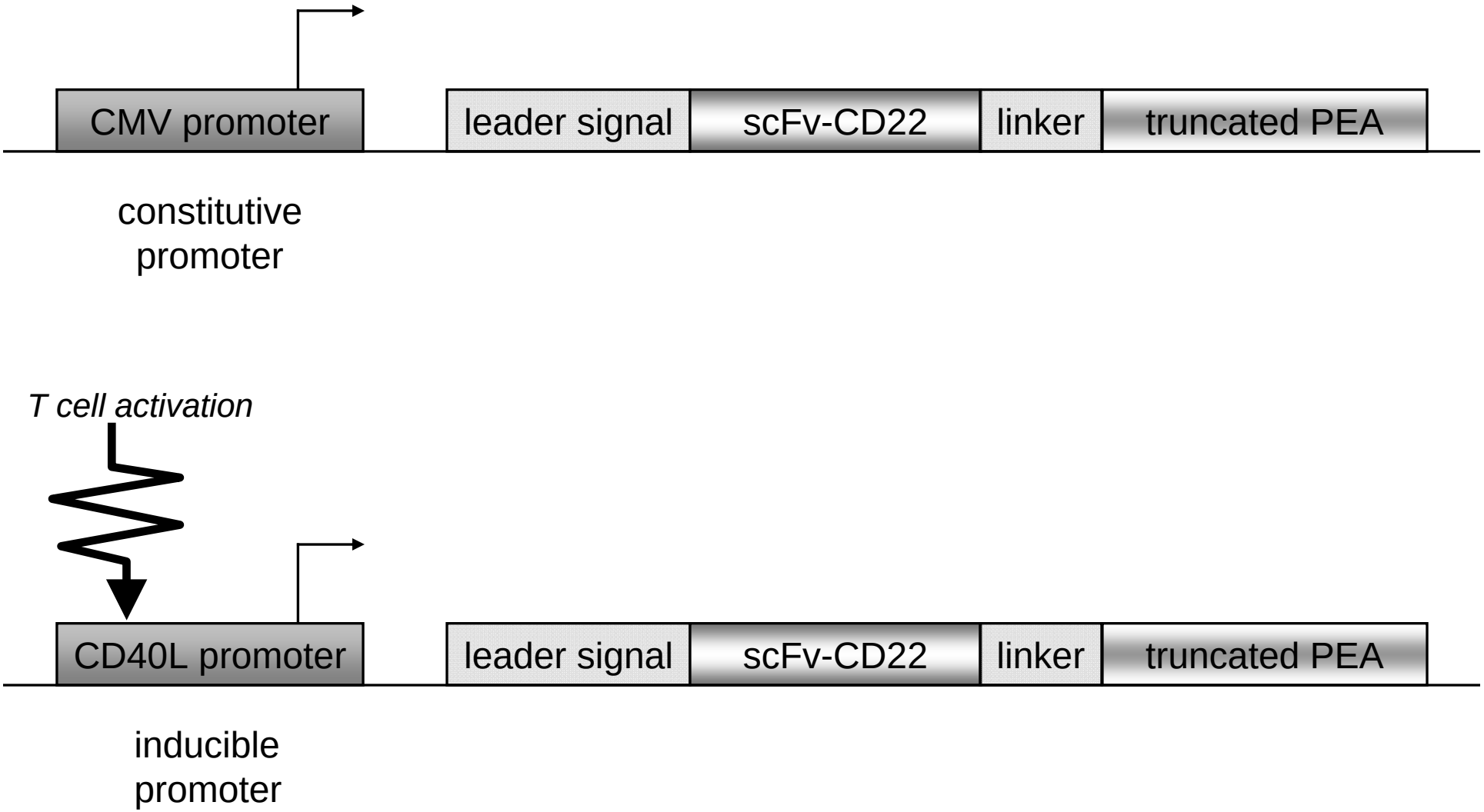
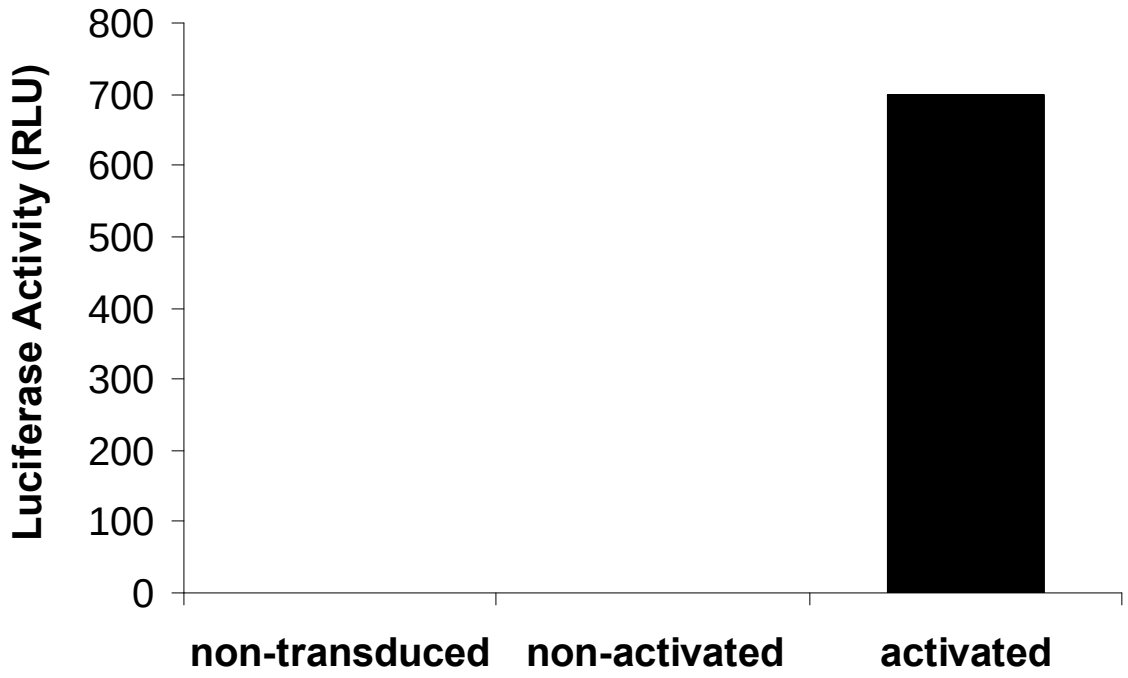

CMV promoter
leader signal
scFv-CD22
linker
truncated PEA
constitutive promoter
T cell activation
CD40L promoter
leader signal
scFv-CD22
linker
truncated PEA
inducible promoter

Figure 2b



Reproduced from graph in grant

Figure 3a

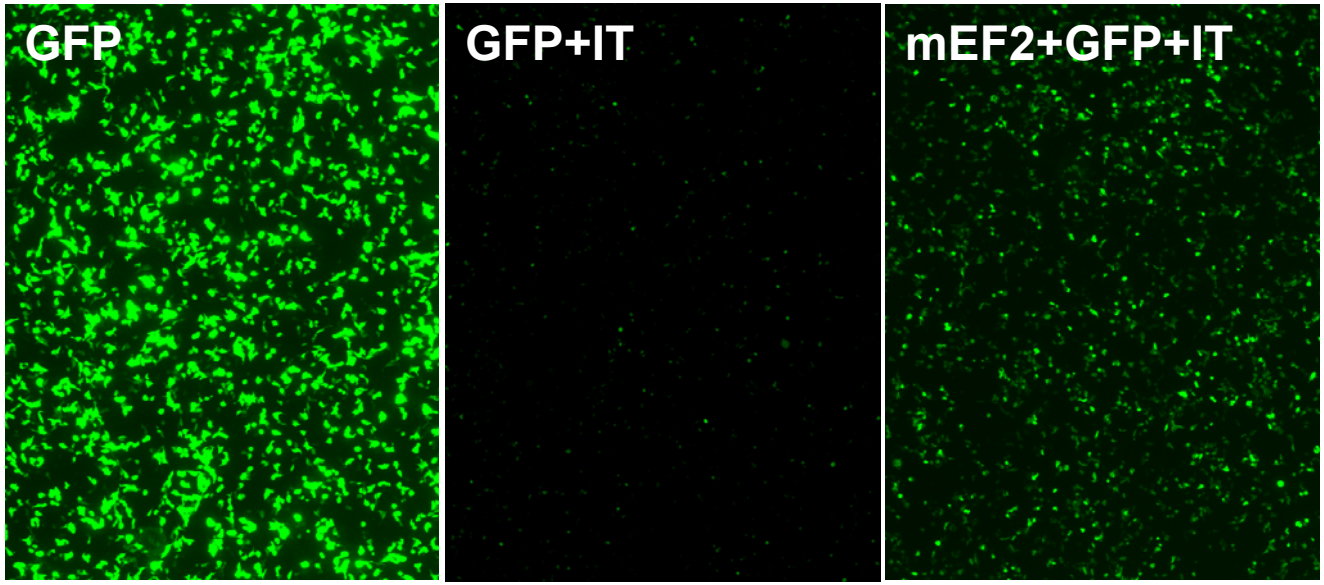


Figure 3b

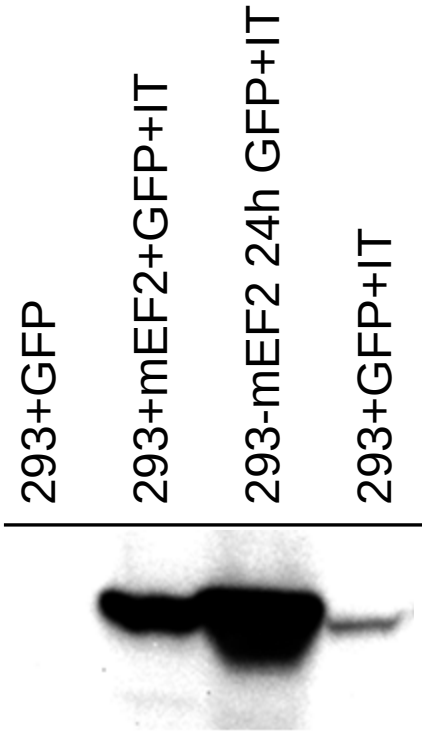


Figure 3c

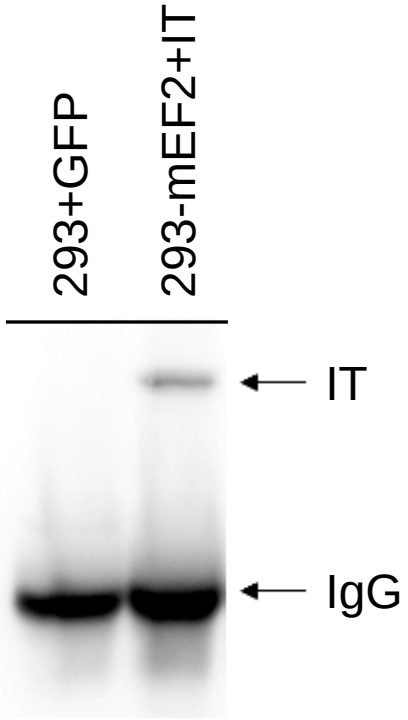


Figure 3d

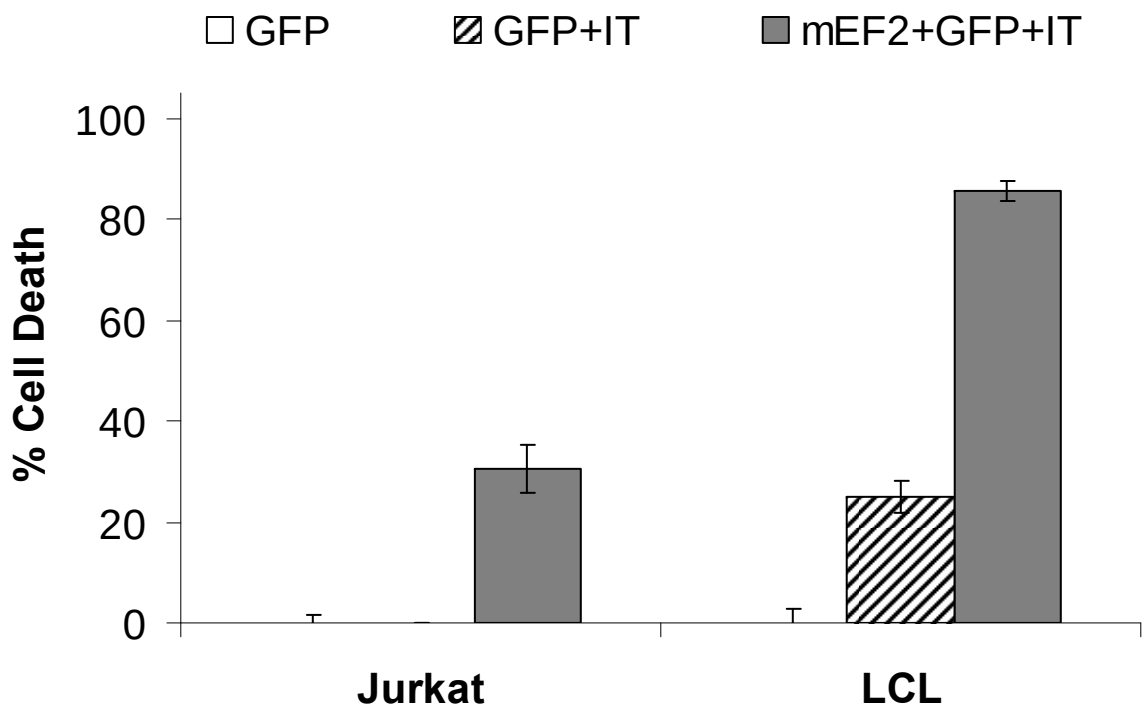


Figure 4a

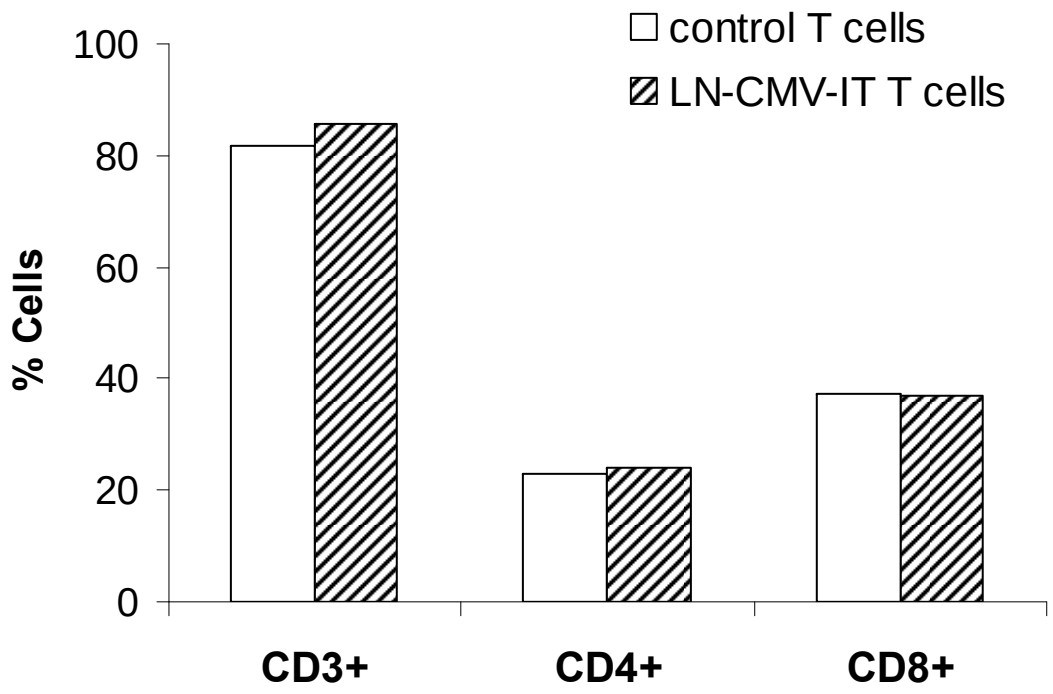

control T cells
LN-CMV-IT T cells
100
80
60
40
20
0
% Cells
CD3+
CD4+
CD8+

Figure 4b

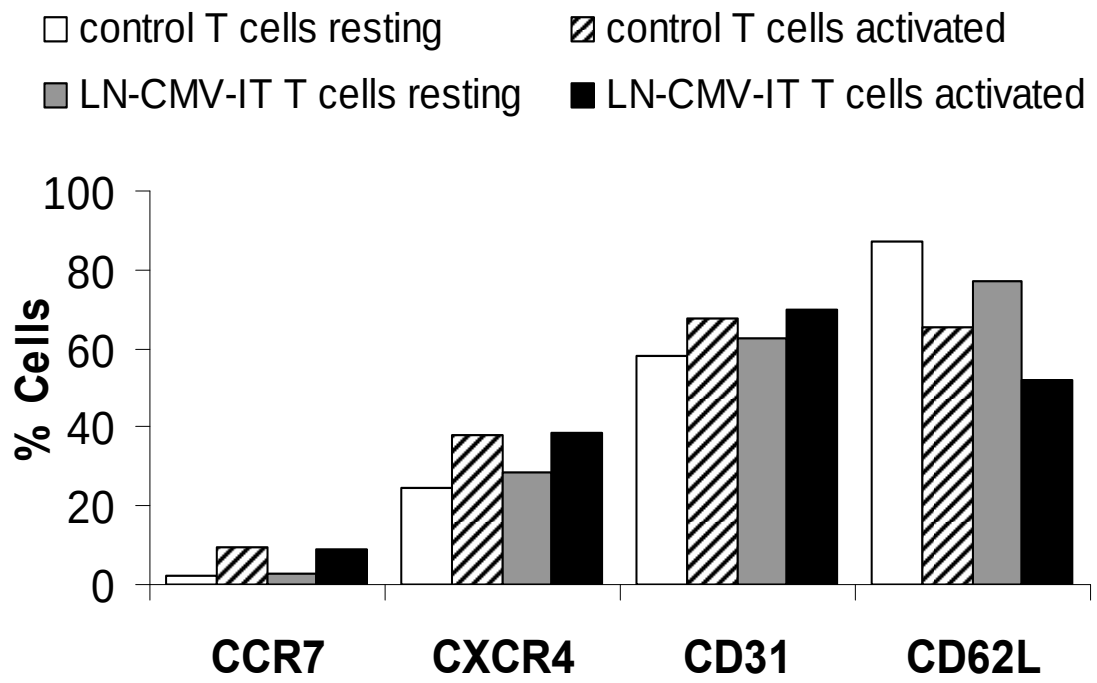

control T cells resting
control T cells activated
LN-CMV-IT T cells resting
LN-CMV-IT T cells activated
100
80
60
40
20
0
% Cells
CCR7
CXCR4
CD31
CD62L

Figure 4c

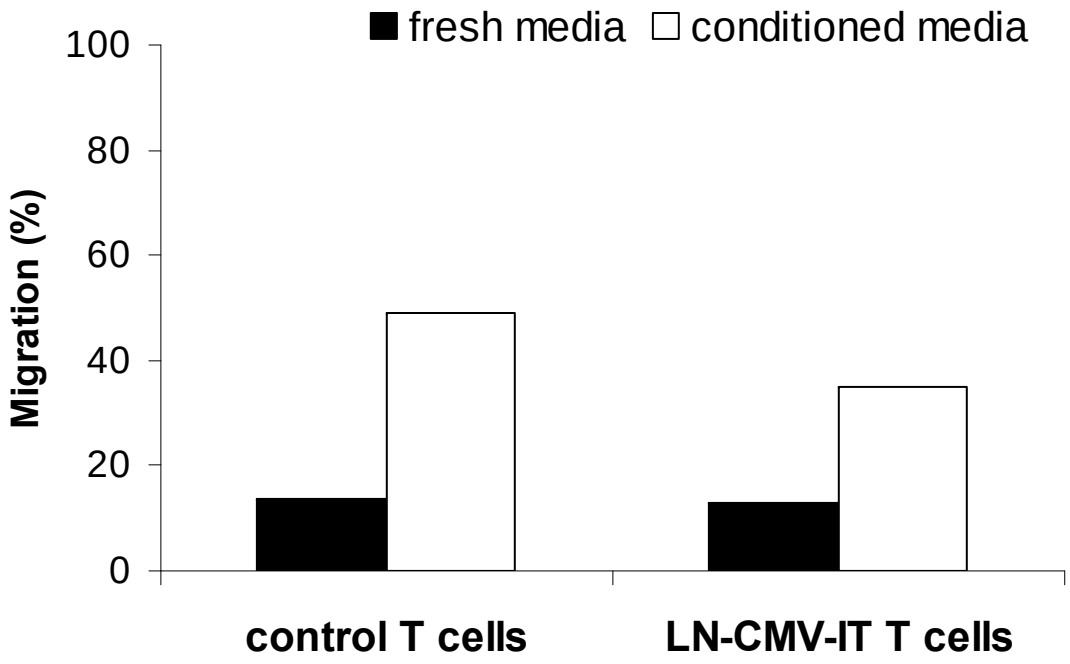

fresh media
conditioned media
100
80
60
40
20
0
Migration (%)
control T cells
LN-CMV-IT T cells

Figure 4d

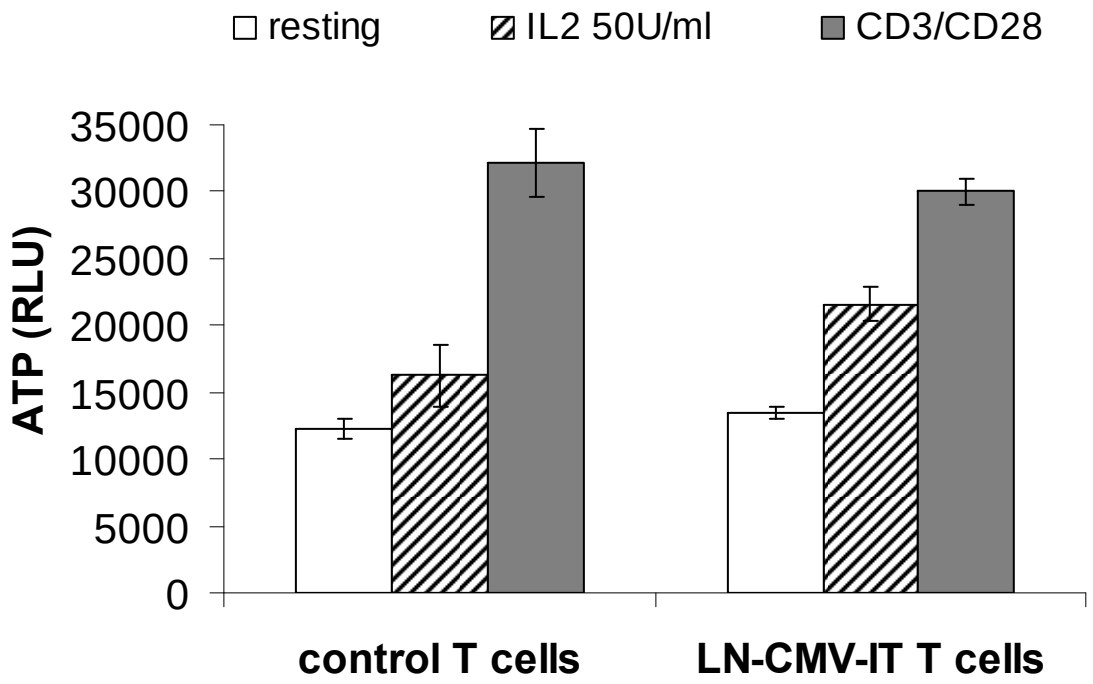

resting
IL2 50U/ml
CD3/CD28
ATP (RLU)
35000
30000
25000
20000
15000
10000
5000
0
control T cells
LN-CMV-IT T cells

Figure 4e

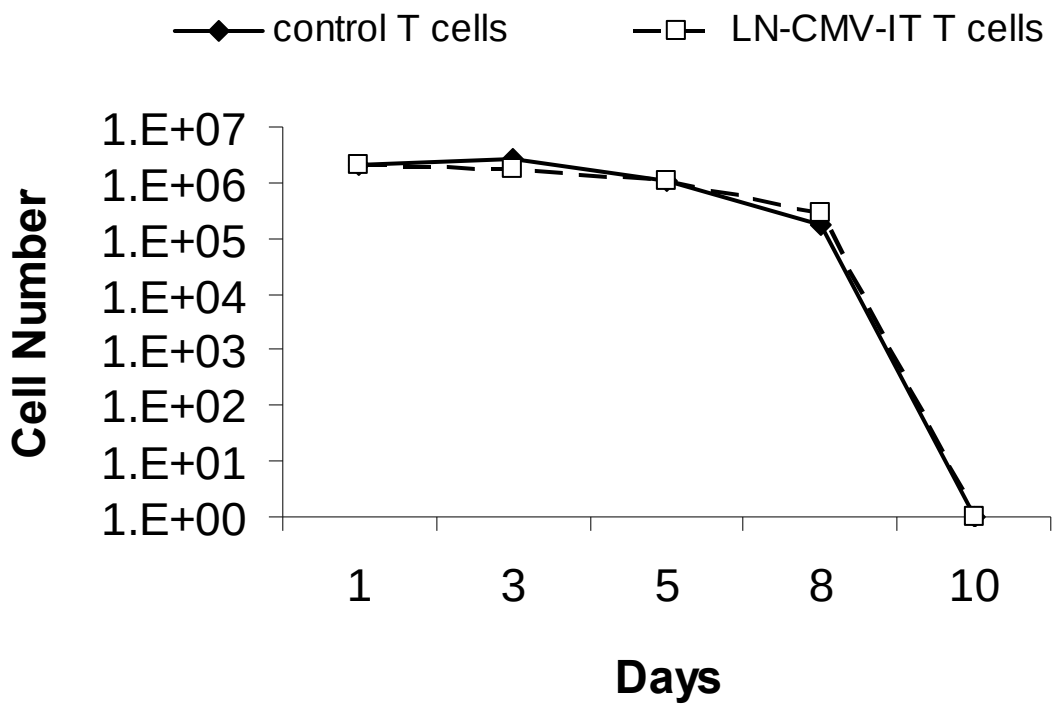

control T cells
LN-CMV-IT T cells
Cell Number
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1
3
5
8
10
Days

Figure 4f

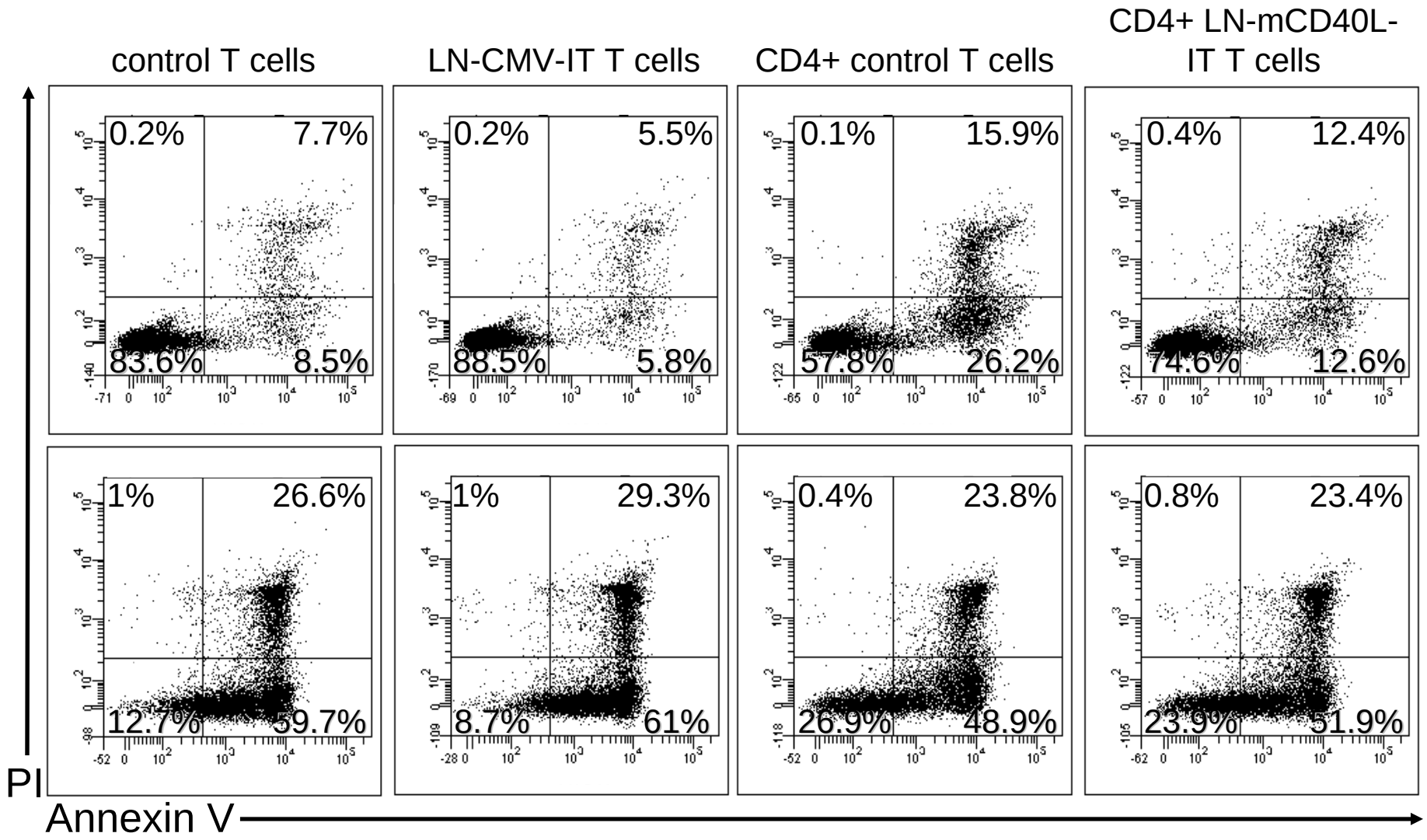

control T cells
LN-CMV-IT T cells
CD4+ control T cells
CD4+ LN-mCD40L-IT T cells
Resting
Activated
PI
Annexin V
0.2%
7.7%
83.6%
8.5%
0.2%
5.5%
88.5%
5.8%
0.1%
15.9%
57.8%
26.2%
0.4%
12.4%
74.6%
12.6%
1%
26.6%
12.7%
59.7%
1%
29.3%
8.7%
61%
0.4%
23.8%
26.9%
48.9%
0.8%
23.4%
23.9%
51.9%

Figure 4g

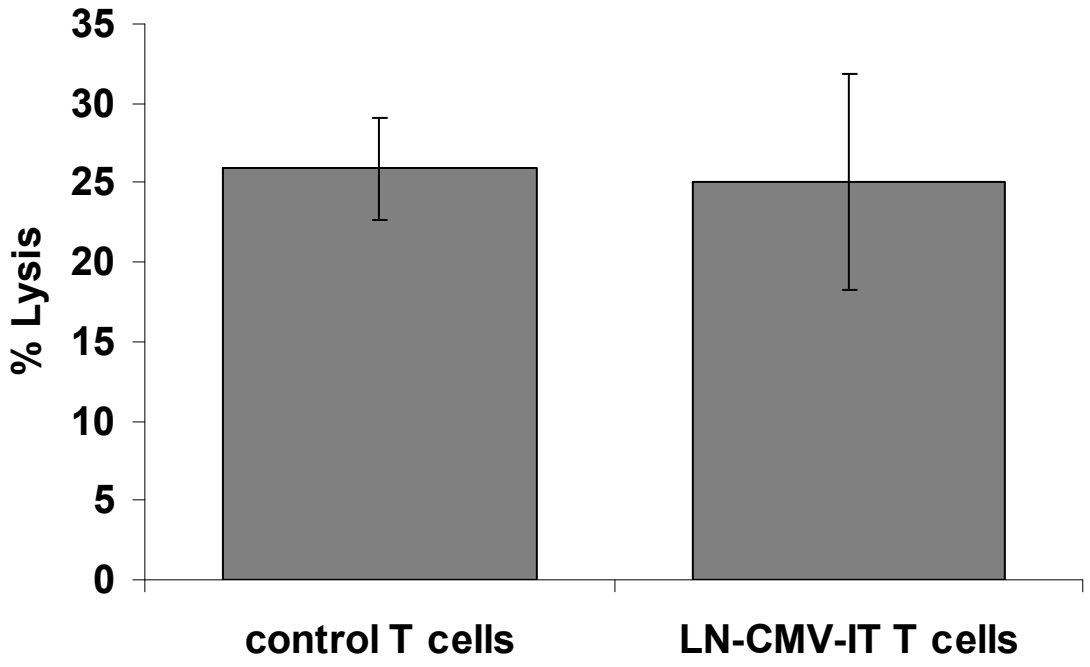


Figure 5a

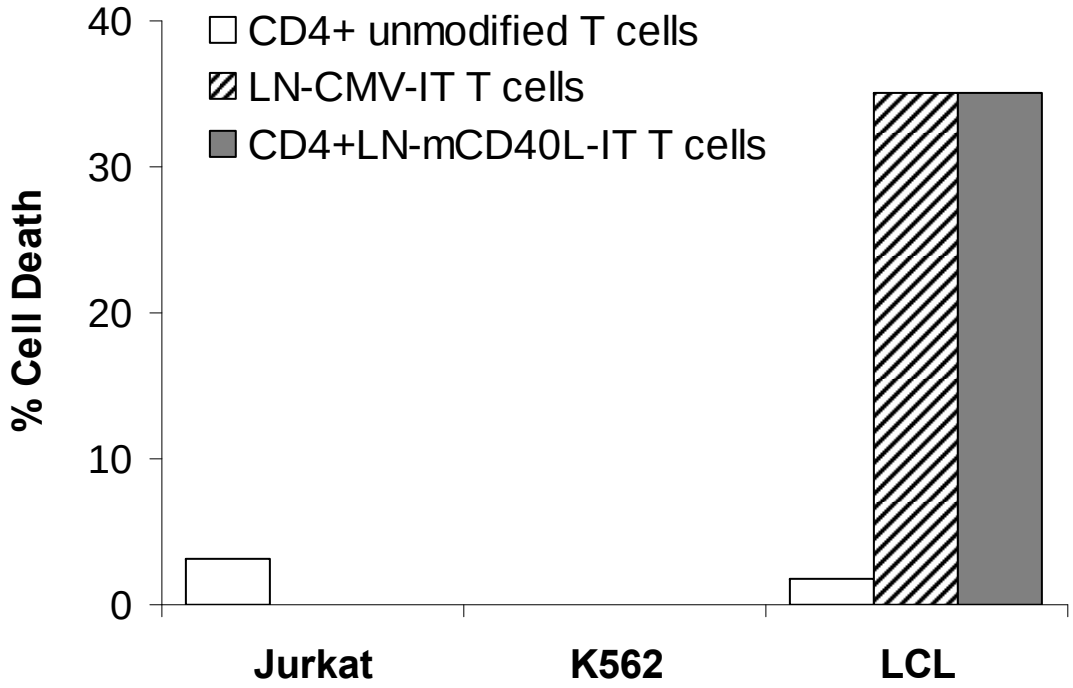

40
30
20
10
0
% Cell Death
CD4+ unmodified T cells
LN-CMV-IT T cells
CD4+LN-mCD40L-IT T cells
Jurkat
K562
LCL

Figure 5b

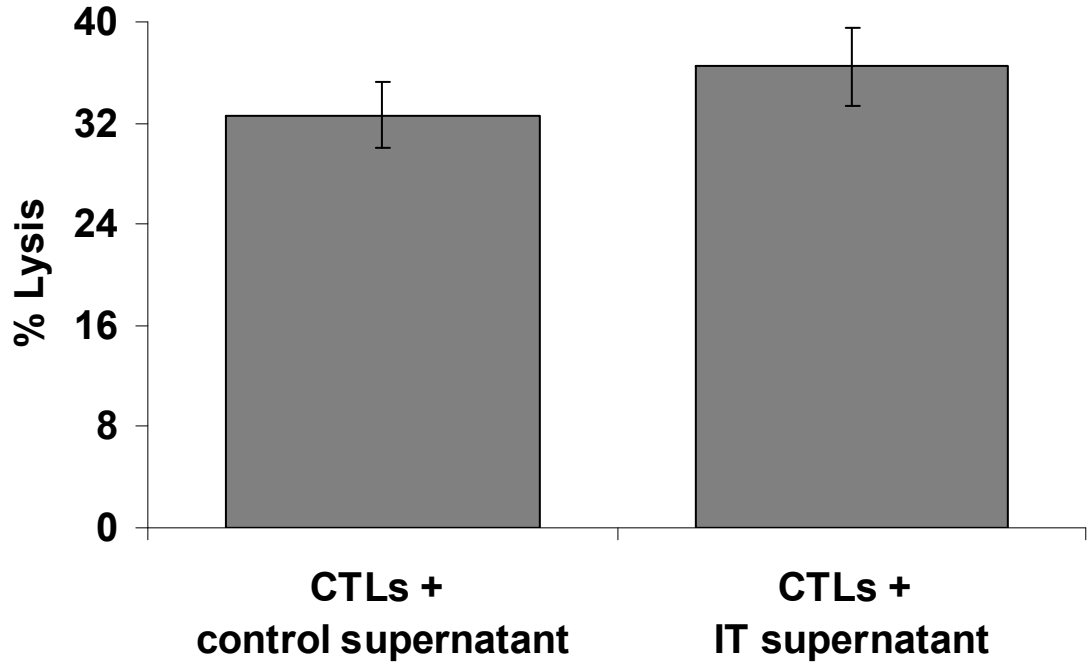
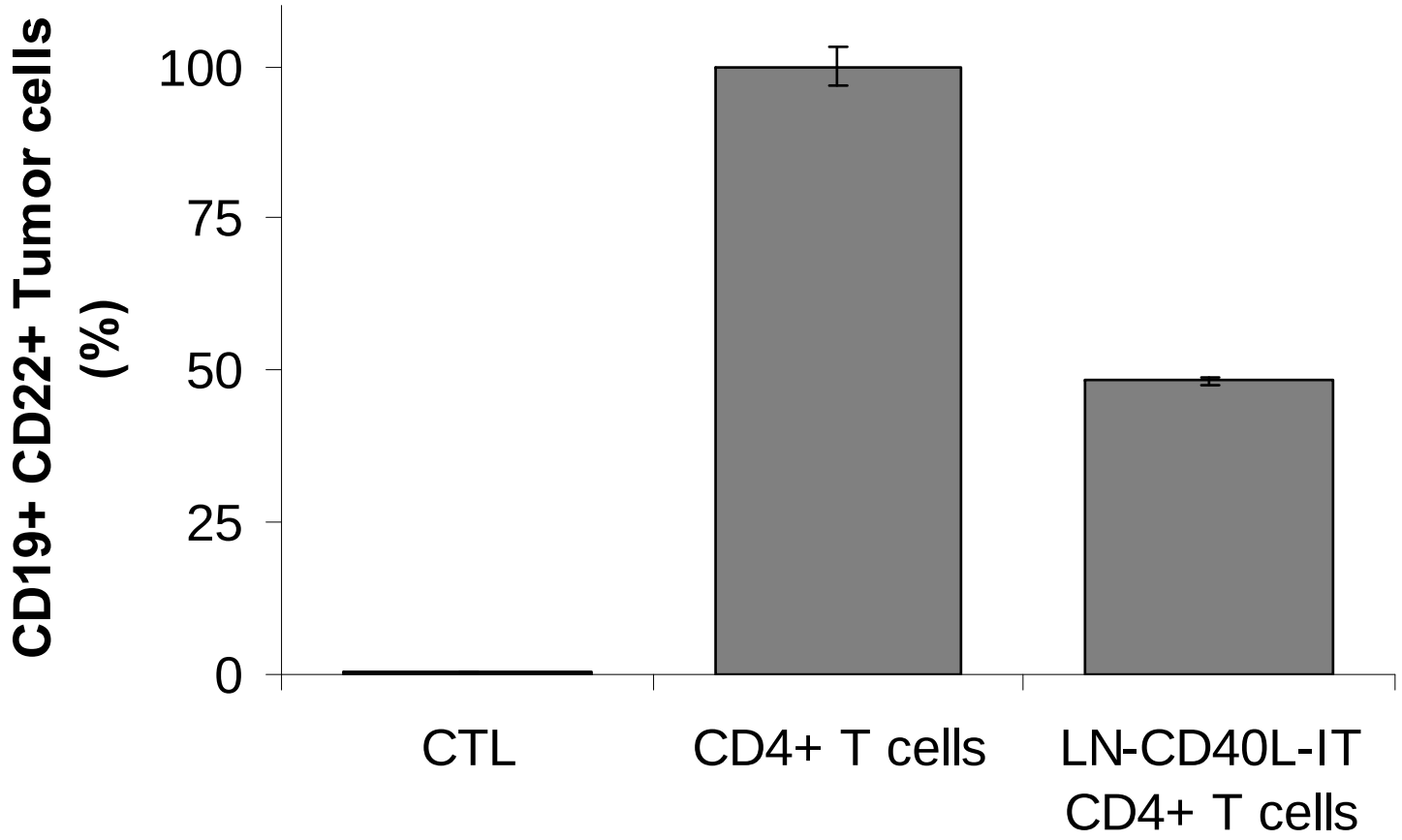

40
32
24
16
8
0
% Lysis
CTLs +
control supernatant
CTLs +
IT supernatant

Figure 5c


CD19+ CD22+ Tumor cells (%)
100
75
50
25
0
CTL
CD4+ T cells
LN-CD40L-IT CD4+ T cells